*CHANGES IN THE PHYSICAL STATE OF DNA DURING THE REPLICATION CYCLE**

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According to the original hypothesis of Watson and Crick[1] each strand of the DNA double helix serves as a template for the synthesis of a new complementary strand of opposite polarity. The observations of Meselson and Stahl[2] on the replication of DNA in *E. coli*, as well as the *in vitro* studies of Kornberg and co-workers,[3] have been entirely consistent with this hypothesis. However, studies with highly purified preparations of DNA polymerase have served to underscore a problem which was raised by Watson and Crick in their original formulation. (1) The strands of the DNA double helix are hydrogen bonded to each other and are not free to act as templates for replication. (2) DNA in its native double helical state is not an effective template for the synthesis of new DNA by highly purified preparations of polymerase.[4, 5]

Watson and Crick discussed the theoretical problem of the unwinding and separation of the strands. They suggested that unwinding might occur simultaneously with replication, synthesis starting at one end of a DNA molecule and continuing along its length. Our observations, on the contrary, suggest that the native helical configuration of DNA is altered at some time *prior* to replication, allowing the strands to act as templates for the synthesis of new DNA.

Observations on Satellite DNA Bands.—We have observed a satellite DNA band[6] of increased buoyant density and one of decreased buoyant density in studying nucleic acid preparations from exponentially growing bacteria at sedimentation equilibrium in CsCl.

(1) *Heavy satellite band:* A small satellite band with the buoyant density characteristic of denatured DNA is present in addition to the principal DNA band in the ultraviolet absorption photographs of Figure 1. We have observed this heavy satellite band on centrifugation of nucleic acid preparations[7] from exponentially growing[8] cultures of *E. coli*, *B. subtilis*, and *B. megaterium*.

Although the DNA of the satellite band resembles denatured DNA in its buoyant density and in its titration behavior in alkaline CsCl (Fig. 1, II *b*), it does not appear to be single stranded. This conclusion has been derived from density labeling experiments in which 5-bromouracil completely replaces thymine in one of the two conserved subunits[2] of DNA. Such half-substituted molecules are called "hybrid" DNA and have a density intermediate between unsubstituted and fully substituted molecules. Satellite bands have been observed corresponding to each of the three native DNA bands, unsubstituted, hybrid, and fully substituted. The satellite band corresponding to native hybrid DNA clearly does not contain single stranded DNA as this would be found either at the density of unsubstituted or fully substituted denatured DNA.

(2) *Light satellite band:* A satellite band of decreased buoyant density has been detected *within* the principle DNA band by ammonium sulfate fractionation of our

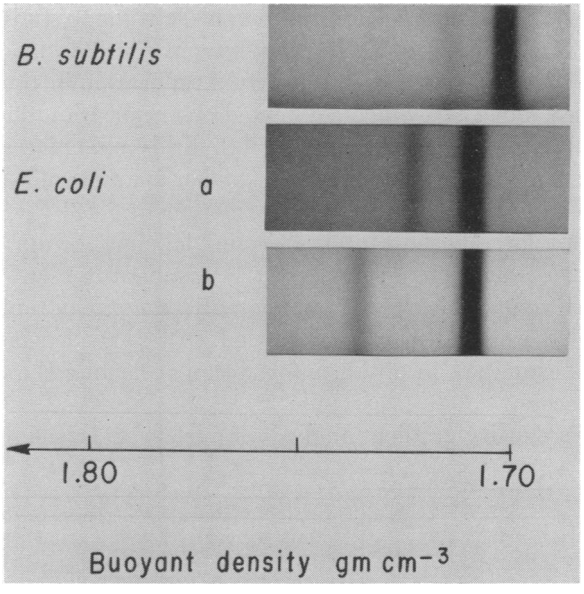

FIG. 1.—Ultraviolet absorption photographs of bands of bacterial DNA in 57.5 weight per cent CsCl solution after 24 hr of centrifugation at 44,770 rpm and 25°C. Bacteria were grown exponentially at 37°C in broth or in a supplemented glucose salts medium with aeration. Medium shifts were carried out using a coarse porosity collodion filter. Nucleic acids were isolated by a modified Kirby method[7] and conditions of ultracentrifugation and absorption photography are described elsewhere.[16] I. DNA isolated from an exponentially growing culture of *B. subtilis* II. (*a*) DNA from a culture of *E. coli* 15 T-A-U[9] starved for thymine for a period of three generations. A similar pattern is observed when DNA is obtained from exponentially growing cultures of this organism. (*b*) Sample as in II*a* but in CsCl solution buffered at pH 10.2 with 0.05 *M* phosphate. The observed increase in density of the satellite band is due to binding of Cs^+ by the polynucleotide, and indicates that the N1 protons of thymine and guanine are being titrated at the high pH. This effect has been described for denatured DNA but is not shown by native DNA.[17]

nucleic acid preparations. When one-half volume of saturated ammonium sulfate in 0.1 *M* sodium citrate is added to the nucleic acid preparation in the cold, a precipitate is formed which contains 10–20% of the total DNA of the preparation. Analytical density-gradient centrifugation of samples of the precipitate and supernatant reveals gross differences in the DNA band patterns of the two fractions. The pattern for the supernatant shows essentially only the principal DNA band. The pattern for the precipitate shows two satellite bands: (*a*) the satellite band of increased density is present as a minor component (heavy satellite band); (*b*) a very sharp band with a modal density slightly *less* (density difference 0.003 gm cm^{-3}) than that of the principal DNA band[10] is present as the major component (light satellite band). This ultraviolet absorption pattern corresponds closely with patterns of radioactive labeling which will be described below, suggesting that DNA which is identifiable by pulse radioactive labeling is precipitable under these conditions.

DNA Replication and the Satellite Bands.—Pulse radioactive labeling studies have shown that DNA molecules in the earliest stages of replication are found in at least two specific density regions. One of these regions corresponds to the denser satellite band, another is contained within the region of the principal DNA band and corresponds to the light satellite band. The experiments were carried out as follows, using

density labeling with 5-bromouracil and radioactive labeling to study DNA molecules at *different stages in their replication.* (1) Experiments were designed to label radioactively *only* those molecules which had initiated replication in the presence of the density label. The molecules which completed one replication during the labeling period are half-substituted with 5-bromouracil (hybrid) and of intermediate density. Those at earlier stages of replication contain smaller amounts of 5-bromouracil than hybrid molecules and have a smaller density increment. (2) Radioactive labeling was carried out for relatively short intervals (pulse labeling) comprising a small fraction of the time necessary to convert all of the unsubstituted DNA to hybrid. In this way we hoped to maximize the proportion of *incompletely* replicated molecules among those which were radioactively labeled. (3) The density distribution of radioactivity was examined to obtain information regarding the earliest stages in the replication process.

Figure 2 shows a density-gradient analysis of nucleic acids isolated from a culture

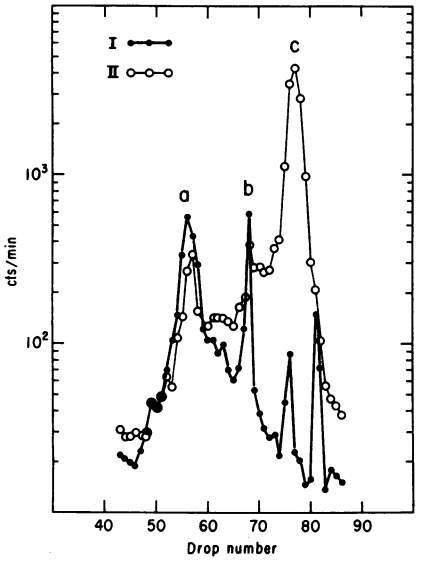

FIG. 2.—Analysis of density labeled and radioactively labeled DNA from *E. coli* 15 T-A-U. Bacteria were first grown exponentially at 20°C in the presence of 14.8 μC/μM (C^{14})-thymine for several generations, then were filtered, washed, and transferred to a 5-bromouracil containing medium. After 10 min $(P^{32}O_4)^{-3}$ was added to a specific activity of 10 μC/μM. After 20 min of P^{32} labeling growth was stopped with 0.01 *M* NaN_3 and the nucleic acids were isolated. For preparative density gradient centrifugation[18] a sample containing 2 μg of DNA in 0.5 ml CsCl solution of density 1.81 gm cm^{-3} was layered beneath 3 ml of CsCl solution (density 1.72 gm cm^{-3}, buffered at pH 8.6 with 0.02 *M* tris) in a lusteroid centrifuge tube. Density fractions were collected through a pinhole in the bottom of the tube after centrifugation for 50 hr at 36,000 rpm and 25°C. For assay of radioactivity in DNA, the fractions were incubated with 0.5 *M* KOH at 37°C for 24 hr to hydrolyse P^{32} labeled RNA to mononucleotides. Carrier calf thymus DNA (Worthington, 50 μg/ml) was added and the DNA was precipitated with cold 5 per cent trichloroacetic acid (TCA), collected on HA millipore filters, and washed 2× with cold 3 per cent TCA. The filters were dried and counted in the Tricarb Scintillation Spectrometer, utilizing the two channels (I, II) as follows. I. The distribution of P^{32} cpm in pulse labeled DNA. II. The distribution of C^{14} cpm in uniformly prelabeled DNA plus 0.35 times the activity in I. (*a*) Hybrid 5-bromouracil band. (*b*) Heavy satellite band. (*c*) Unsubstituted DNA band.

of *E. coli* 15 T-A-U[9] shortly after transfer from normal to 5-bromouracil containing medium. Only a small amount of DNA has been converted to hybrid (band *a*), about 10% is present in the heavy satellite band (band *b*), and the rest is found at the density of unsubstituted DNA (band *c*). Pulse labeled DNA synthesized shortly *after* the transfer is indicated by P^{32} activity which remains acid precipitable after the extensive alkaline treatment used to hydrolyse the labeled RNA. Its density distribution will be considered in some detail.

(1) *Pulse labeled DNA occurs in the region of the unsubstituted DNA band, but appears to be physically distinct from the bulk of the DNA of this band:* If pulse labeled DNA were physically associated with the bulk of the unsubstituted DNA, the density gradient distribution for the two kinds of DNA should not be grossly dis-

similar in the region of the unsubstituted DNA band. However, pulse labeled DNA occurs in two, distinct, narrow peaks within the DNA band and otherwise is essentially absent in this density region. The lightest of these two peaks corresponds in density to the mode of the light satellite band precipitable with ammonium sulfate. The narrowness of the peaks of pulse labeled DNA is consistent with the sharpness of the light satellite band observed after ammonium sulfate fractionation. It indicates that the labeled DNA has a significantly higher effective molecular weight in the CsCl gradient than the bulk of the DNA of the principal band. This could result from selective aggregation between pulse labeled DNA molecules of the same density, or might reflect a selective resistance to shear fragmentation of large chromosomal segments[11] which are pulse labeled. In accord with the latter possibility Goldstein and Brown[12] have demonstrated that pulse labeled DNA is selectively resistant to sonic fragmentation.

(2) *Pulse labeled DNA is physically associated with the DNA of the heavy satellite band:* In the region of the heavy satellite band the pulse labeled DNA concentration increases sharply, forming a large peak. The narrowness of this radioactive peak, like that of the peaks in the region of the unsubstituted DNA band, may be due either to selective aggregation or to shear resistance of the labeled molecules. There is a more gradual increase in the concentration of pulse labeled DNA between the position of the heavy satellite band and that of the hybrid DNA band, where a second large peak is found. In a number of experiments under conditions of high ionic strength ($\mu > 1$) and DNA concentration (> 20 μg/ml) a decrease of density of the heavy satellite band was observed, suggesting that the DNA of the satellite band readily "renatures."[13, 14] Under these conditions, when the DNA of the heavy satellite band has shifted to a region of lower density, the pulse labeled DNA shifts *in the same manner.*

(3) *The pulse labeled DNA in the region of the unsubstituted DNA band and the heavy satellite band consists of DNA molecules in early stages of replication:* Pulse labeled DNA molecules in late stages of replication would be expected to be found at or near the density of hybrid DNA, as all of the pulse labeled molecules initiated replication in the presence of 5-bromouracil. It might be that the finding of pulse labeled DNA in the region of the unsubstituted DNA band and heavy satellite band is an artifact, due to aggregation between pulse labeled hybrid DNA and unsubstituted DNA. This artifact is unlikely because the concentrations of DNA employed in density gradient analysis were very low (0.6 μg/ml) and because the radioactivity peaks do not correspond to positions of maximum DNA concentration in the density gradient. Two experimental observations support the conclusion that the pulse labeled DNA in the region of the unsubstituted DNA band and heavy satellite band consists of molecules in early stages of replication: (*a*) Pulse labeled DNA may be chased away from these regions by allowing further incorporation of 5-bromouracil in the absence of radioactive labeling. (*b*) Pulse labeled DNA is found selectively in the satellite band regions *even when the labeling is carried out in the absence of 5-bromouracil.*

Figure 3 illustrates the first of these two observations and shows the density region of the unsubstituted DNA band before (3*a*) and after (3*b*) a chase in nonradioactive, 5-bromouracil containing medium. Hybrid DNA bands have been omitted from the figure to facilitate comparison of the pulse labeled DNA distributions in the region

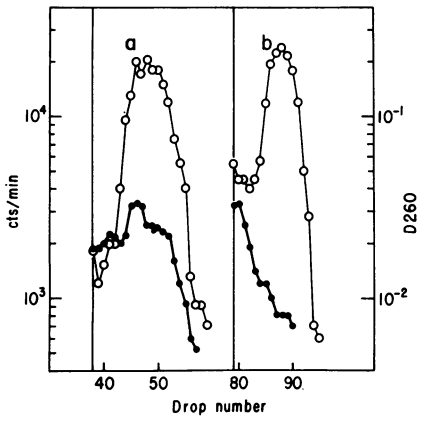

FIG. 3.—Chase of pulse P^{32} labeled DNA from the region of the unsubstituted DNA band by continued density labeling in a nonradioactive medium. (*a*) Density distribution of pulse P^{32} labeled DNA in the region of the unsubstituted DNA band before the chase with cold phosphorous. (*b*) Density distribution of pulse P^{32} labeled DNA in the same region as shown in (*a*) but after the chase with cold phosphorous. Exponentially growing bacteria (20° C, generation time 120 min) were filtered, washed, and resuspended in a 5-bromouracil containing medium for 15 min. $(P^{32}O_4)^{-3}$ was added to a specific activity of 100 μC/μM (4 μg/ml P) and an aliquot of culture was harvested after 30 min of radioactive labeling. A second aliquot was filtered, washed, resuspended in a nonradioactive 5-bromouracil containing medium with 200 μg/ml P, and harvested after 120 min in the nonradioactive medium. The nucleic acids were isolated from each sample, and treated with ribonuclease (Worthington, 10 μg/ml). After dialysis in the cold for 40 hr against 0.5 *M* NaCl, 0.015 *M* sodium citrate the nucleic acids were studied by preparative density gradient centrifugation. 80 μg DNA was used, and density fractions were plated on aluminum planchets, dried, and counted in a thin window gas counter (Nuclear Chicago) after dilution to a volume of 0.5 ml and measurement of absorption at 260 mμ (D260). ●, P^{32} cpm. O, D260.

of interest. Higher DNA concentrations (20 μg/ml) were employed in this experiment and, as may be seen in Figure 3*a*, under these conditions the pulse labeled DNA of the heavy satellite band has renatured and is found to overlap the unsubstituted DNA band. After removal of the radioactivity, incorporation of 5-bromouracil was allowed to continue until one-third of the originally unsubstituted DNA was converted to hybrid. Figure 3*b* shows that more than 70 per cent of the pulse labeled DNA was displaced from the region of the unsubstituted DNA band by the further incorporation of 5-bromouracil.

The results of a pulse labeling experiment *not* involving 5-bromouracil substitution are shown in Figure 4. To achieve conditions of very limited DNA synthesis during pulse labeling, cells were thymine starved for a period which was too short to

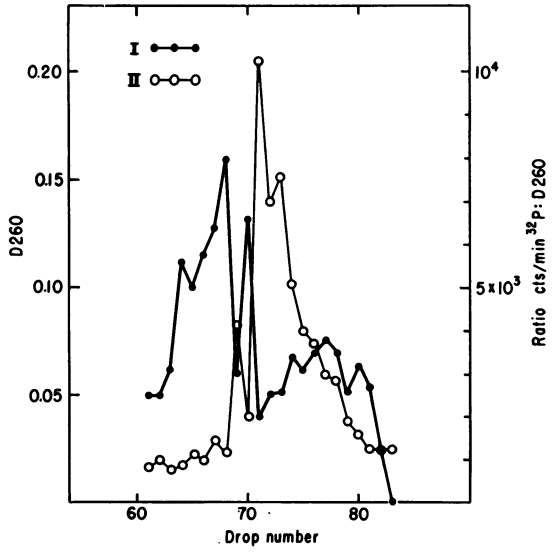

FIG. 4.—Density gradient analysis of DNA from *E. coli* 15 T-A-U labeled with $(P^{32}O_4)^{-3}$ in a thymineless medium. I. The ratio of cpm P^{32} above background to the optical density at 260 mμ. This is a lower bound for the specific activity of the DNA. II. Optical density at 260 mμ (D260). Bacteria growing exponentially at 20°C were filtered, washed, and resuspended in a thymineless medium and $(P^{32}O_4)^{-3}$ was added to 2 μC/μM. After 20 min growth was stopped and the bacterial nucleic acids were isolated. Ribonuclease (Worthington, 10 μg/ml) was added to the nucleic acid preparation, which was then dialysed 40 hr against 0.5 *M* NaCl, 0.015 *M* sodium citrate in the cold. Preparative density gradient analysis was carried out as described for Figure 3 except that 20 μg DNA was used. Similar results were obtained when pulse labeling was carried out with a 100-sec supply of (C^{14})-thymine, instead of P^{32}.

allow any loss of viability. After labeling, density gradient analysis revealed that the DNA of highest specific activity was found in a region of increased density, corresponding to the heavy satellite band, and in a second region on the light side of the main DNA band, corresponding to the light satellite band. When the pH of the banding solution was increased to 10.2, high specific activity DNA extended to regions of even greater density, consistent with the expected shift of the heavy satellite band at the higher pH. Experiments of this kind are in agreement with the studies described above; they confirm the specific changes in buoyant density found for actively replicating DNA molecules.

Discussion.—From the work of Meselson and Stahl[2] it is known that few molecules of the total DNA complement of *E. coli* are actively replicating at any given time during the division cycle. We have presented evidence suggesting that a significant proportion of the total DNA of *E. coli* may be isolated in states differing from native DNA in buoyant density and that *molecules in early stages of replication are found in these states rather than in the "native" state.* Taken together, these observations suggest that preparatory to replication DNA enters a state in which it differs structurally from native DNA. It is tempting to try to infer some of the characteristics of this *in vivo* state from the properties of the DNA after isolation.

After density gradient centrifugation, two DNA components of unusual density are found. The component which has been more extensively studied forms a distinct band of increased density relative to the bulk of the DNA (heavy satellite band). These experiments suggest that it is a collapsed structure in which the conserved subunits are not dissociated. The other component is found at a position overlapping that of the bulk of the DNA, but at a slightly reduced density (light satellite band). Experiments in progress to characterize the latter component have indicated that it is unstable and subject to low recovery during density-gradient centrifugation. Its reduced density and its precipitability with ammonium sulfate suggest that it may be associated with specific proteins, possibly polymerases[15] and nucleases. It is possible that the observed differences between these two unusual component DNA's are merely a consequence of the isolation procedure. Conversely, they may reflect significant differences in stability between different classes of DNA molecules in a "prereplication" state.

The collapse of the component which gives rise to the heavy satellite band suggests that it does not have the ordered helical structure postulated by Watson and Crick. This low stability might be brought about by a reduction of the base-base Van-der-Walls interactions or by an interference with the hydrogen bonding between the strands. In any case, the strands of such a destabilized structure would be available to act as templates more readily than would the strands of "native" DNA.

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[6] Other workers have also observed satellite bands in studying DNA from a variety of sources. Ephrussi-Taylor (*J. de Chim. Phys.*, **58**, 1090 (1961)) working with synchronized *D. pneumoniae*, and Lark, Cavalieri, and Rosenberg (unpublished observations, 1962) studying synchronized *A. faecalis*, have observed satellite bands of increased buoyant density. With animal cell DNA, satellite bands both of increased and of decreased density have been reported (Kit, S., *J. Mol. Biol.*, **3**, 711 (1961); Sueoka, N., *J. Mol. Biol.*, **3**, 31 (1961); Schildkraut, C., J. Marmur, and P. Doty, *J. Mol. Biol.*, **4**, 430 (1962)). Bands of decreased density were also reported by Marmur, Rownd, Falkow, Baron, Schildkraut, and Doty (these PROCEEDINGS, **47**, 972 (1961)) with purified DNA from *A. faecalis* and *S. marcesens* following preparative CsCl-density-gradient fractionation.

[7] When bacterial DNA is isolated by other standard procedures (e.g., Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961)) or by density-gradient centrifugation of bacterial lysates[2] the satellite band is not observed. Our isolation procedure utilizes deproteinization with phenol (Kirby, K. S., *Biochem. J.*, **66**, 495 (1957)) and ammonium sulfate-isopropanol precipitation of the nucleic acids. It will be described in detail elsewhere. The heavy satellite band is not an artifact of preparation, as it has been verified experimentally that native DNA is not denatured by our isolation procedure.

[8] With saturated cultures, or cultures in which DNA synthesis has run to completion after a shift to a medium lacking an essential amino acid (or after a shift from a medium containing amino acids to a minimal medium),[9] the satellite band is absent or greatly reduced in amount. However, if DNA synthesis in *E. coli* 15 T-A-U is stopped by shifting an exponentially growing culture to a thymineless medium the satellite band is observed to be undiminished (Fig. 1).

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*THE EFFECT OF SECONDARY STRUCTURE ON THE TEMPLATE ACTIVITY OF POLYRIBONUCLEOTIDES**

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Current experiments on the synthesis of polynucleotides and proteins seem to us to emphasize the functional importance of polynucleotide secondary structure. Thus, for example, the highly ordered double-helical structure of DNA[1] readily leads to ideas concerning DNA replication.[2, 3] Although our notions concerning the